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HUMAN EPITHELIAL CELL ACTIVATION SYSTEMS

by

J. Justin McCormick
Carcinogenesis Laboratory
B609 W. Fee Hall
Michigan State University
East Lansing, MI 48824

R8055630

Project Officer

Michael Waters
Genetic Toxicology Division
Health Effects Research Laboratory
Research Triangle Park, North Carolina 27711

HEALTH EFFECTS RESEARCH LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
RESEARCH TRIANGLE PARK, NORTH CAROLINA 27711

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16. ABSTRACT

Much effort has been placed on the development of short-term assay systems that use the induction of mutations in bacterial and mammalian cells as the basis for predicting the carcinogenicity of environmental chemicals. However, evidence has accumulated that suggests that most environmental chemicals require enzymatic activation to produce metabolites that will react with cellular macromolecules, and many or the bacterial and mammalian cell lines identified are unable to produce that activation. Consequently, scientists began to use microsomal systems to supply activation.

In this study, two short-term assays were examined and cells that appeared capable of metabolizing various carcinogens were identified. In the first method, the metabolism of tritiated benzo(a)pyrene [B(a)P] to aqueous-acetone soluble products was measured. While this method is useful to identify cells that are capable of metabolizing B(a)P or related compounds, it is not applicable to other classes of compounds. Therefore, a second, more general method was examined. This second method detects the formation of agents that damage DNA by measuring DNA synthesis inhibition after exposure to a carcinogen.

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The complexities of environmental problems originate in the deep interdependent relationships between the various physical and biological segments of man's natural and social world. Solutions to these environmental problems require an integrated program of research and development using input from a number of disciplines. The Health Effects Research Laboratory conducts a coordinated environmental health research program in inhalation toxicology, genetic toxicology, neurotoxicology, developmental and experimental biology, and clinical studies using human volunteer subjects. Thes studies address problems in air pollution, water pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides and other chemical pollutants. The Laboratory participates in and provides data for the development and revision of criteria documents on pollutants for which national ambient air quality and water quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of environmental regulatory decisions involving the protection of the health and welfare of all U.S. inhabitants.

This study was under takened to examine two short-term assays and to identify cells that appeared capable of metabolozing various carcinogens.

F. Gordon Hueter, Ph.D.

Director

Health Effects Research Laboratory

ABSTRACT

Human cells that appear capable of metabolizing various classes of carcinogens have been identified using one of two methods: metabolism of tritiated benzo-(a)pyrene to aqueous-acetone soluble forms or inhibition of cellular DNA synthesis. Each of the assay systems was optimized and the results on 15 human epithelial cell lines were compared. One or more cell lines were found to activate each of four classes of carcinogens examined: polycyclic hydrocarbons, aromatic amines, heterocyclic hydrocarbons, and nitrosamines. Cells that appeared capable of metabolizing polycyclic hydrocarbons or aromatic amines by these methods were also found to produce metabolites which were cytotoxic to cocultivated human xeroderma pigmentosum fibroblasts after a 48 hr exposure to the carcinogen.

To determine whether mutagenic products were produced, lethally X-irradiated human kidney carcinoma-deprived epithelial cells, which had constant levels of B(a)P-metabolizing activity, were cocultivated with target human skin fibroblasts (XP12BE), which lack excision repair capability for B(a)P-DNA adducts. The optimal conditions determined for the cell-mediated cytotoxicity assay were a 48-hr exposure to B(a)P concentrations ranging from 0.1 to 1 μM at a metabolizing cell: target cell ratio of at least 1:1. Under these conditions, the frequency of mutations to 6-thioguanine resistance induced in the target XP12BE cells by B(a)P as well as the binding of tritium-labeled B(a)P to DNA was also shown to be concentration dependent. High-pressure liquid chromatography analysis of enzymatically degraded B(a)P-DNA adducts revealed two peaks: a major peak (82%) which cochromatographed with the guanosine adduct-standard synthesized from anti-isomeric-7,8-dihydro-diol-9,10-epoxide of B(a)P; and a minor peak (18%) which cochromatographed with the guanosine adduct-standard synthesized from syn-isomeric-7, 8-diol-9, 10-epoxide of B(a)P.

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SECTION I

INTRODUCTION

Since every year new chemicals are introduced into our environment, it is important to have sensitive screening assays available to predict their biological effects. Thus, much effort has been placed on determining the potential carcinogenicity of environmental chemicals by developing short-term test systems which use induction of mutations in bacteria and mammalian cells as their basis for prediction (1). However, evidence has accumulated which suggests that the majority of chemicals require enzymatic activation to produce metabolites which will react with cellular macromolecules. Since many bacteria and mammalian cells used in the short-term test systems are unable to activate these chemicals, microsomal systems are commonly used to supply activation (2-5).

Cell-mediated systems have also been used to provide activation, but to a more limited extent (6-10). However, recent studies have demonstrated that the activation of polycyclic hydrocarbons by subcellular fractions can produce metabolites which will react with DNA but which are not generally produced by intact cells (11-16). The validity of short-term tests, however, is based on the assumption that the DNA adducts formed in the cells utilized for the test are representative of those found in mammals treated with the same parent compound. The results with the polycyclic hydrocarbons suggest that short-term assays utilizing subcellular fractions can be misleading as predictive indicators for at least this class of compounds.

Because the differences in the metabolic activation seem to arise because of cellular disruption, intact cells are to be preferred to subcellular

fractions. Thus, it is important to be able to easily identify cells that are capable of metabolizing these chemicals. One such method which we used is that of Kouri et al. (17) which measures the enzymatic conversion of tritiated BP to aqueous-acetone soluble products. While this assay is useful for the identification of cells capable of the metabolism of BP or related compounds, it is not useful for the identification of cells capable of metabolizing other classes of carcinogens. Therefore, we have examined a second, more general method which detects the formation of agents that damage DNA by measuring DNA synthesis inhibition after exposure to the carcinogen (18). Because we wanted a source of human metabolizing cells with an infinite lifespan and with uniformly high levels of carcinogen-activating enzymes, we first examined human epithelial cell lines derived from tumors. Since these 2 screening assays can provide indications of metabolism, but give unequivocal evidence only of the inability of cells to metabolize, the cells which proved positive by either or both assays were examined further for biological evidence of metabolic activation. This was done by testing various carcinogens for mutagenicity, DNA binding, and/or cytotoxicity in human cells using a cell-mediated assay system with the candidate activating cells as the metabolizing cells (10). We selected XP12BE cells as the target cells for this system because we and others have shown that they are abnormally sensitive to various compounds and UV light, due to the inability to excise DNA damage (19-22). Therefore, these cells can be expected to accumulate DNA damage during the prolonged carcinogen exposure periods. In this report, we describe two screening methods for the identification of human cells that may be able to activate polycyclic hydrocarbons, aromatic amines, nitrosamines, and/or heterocyclic hydrocarbons and the use of these cell lines as metabolizing cells in the human cell-mediated cytotoxicity mutagenicity and transformation assays developed in this laboratory with XP12BE cells as target cells (10). In addition,

we demonstrate the mutagenicity of BP using metabolizing cells selected by these techniques and human fibroblasts as target cells.

SECTION 2

CONCLUSION AND RECOMMENDATIONS

The procedures described here make it possible to find animal or human cell lines which are capable of metabolizing mutagens and/or carcinogens to reactive species. As demonstrated these cells can then be used as feeder layers to produce active metabolites of carcinogens which cause cytotoxicity, mutagenicity or transformation of target cells. In the case of the polycyclic aromatic hydrocarbon, benzo(a)pyrene, we demonstrated that the DNA adducts formed are those found in the DNA of cells that are capable of metabolizing this compound. This is unlike the results found with S9 fractions where the DNA adducts are from different metabolites than is found in cells.

When short term assays first became popular in the middle 1970's, much of the interest necessarily focused on suitable target cells for these assays. In the ensuing years, suitable bacterial, animal and human-cell lines and strains have been developed as target cells and the methods of handling them have become reasonably routine. What has now become more obvious is that the metabolism of mutagens and/or carcinogens is frequently a complex process which can hardly be mimicked by use of a simple S9 fraction as is frequently done. The use of mammalian cells as demonstrated here is a more realistic method of providing such activation. It should be possible to couple such activation systems to even simple mutation assays as the Salmonella test of Ames to provide a more realistic test. It is recommended that workers utilizing short term tests adapt their tests to use mammalian cell activation techniques as an alternative activation technique to the use of S9 fractions.

SECTION 3

METHODS AND MATERIALS

Source of Chemicals

The following chemicals were stored at -20°: BP (Sigma Chemical Co., St. Louis, MO), B(b)F, B(j)F, B(k)F, azapyrene, azachrysene, azabenzanthracene, BPDE 1 (Chemical Carcinogenesis Program, National Cancer Institute), MBOCA (RFR Corp., Hope, RI), DEN (Eastman Organic Chemicals, Rochester, NY), DMN (Aldrich Chemical Co., Milwaukee, WI), AAF, and AABP (generous gifts from Drs. J.A. and E.C. Miller). BP(56 Ci/mmole; Amersham Corp., Arlington Heights, IL) and dThd (64 Ci/mmol; New England Nuclear, Boston, MA) generally labeled with tritium were stored at 4°. Glass-distilled DMSO, hexane, and acetone were purchased from Burdick and Jackson Laboratories, Muskegon, MI.

Cell Culture

Cells. The epithelial cell lines used in these studies were obtained from the Naval Biosciences Laboratory Naval Supply Center, Oakland, California (except for the PC-3 which was a gift from Dr. M.Edward Kaighn). The XP fibroblasts were derived from skin biopsies of an XP patient (XP12BE, complementation group A, obtained from the American Type Culture Collection, Rockville, MD). Normal human fibroblasts were derived from fresh foreskin tissue. All cells were grown at 37° in air atmosphere at 5% CO_2 and 95% humidity.

Culture Medium. Cells were routinely cultured in minimum essential medium (Eagle's) with 50 µg gentamycin (Schering Corp., Kenilworth, NJ) per ml supplemented with either 15% fetal bovine serum (Grand Island Biological Co.,

Grand Island, NY or Sterile Systems, Inc., Logan, UT) for XP cells or 10% fetal bovine serum for NF cells and epithelial cell lines. All cells were stored in liquid N_2.

Freezing Medium. Cells were suspended in minimum essential medium (Eagle's supplemented with 17% fetal bovine serum, 50ug gentamycin per ml, and 10% glass-distilled DMSO.

Hydrocarbon-Metabolizing Activity Assay. The hydrocarbon-metabolizing activity of the cell lines was determined by measuring their ability to convert tritium labeled BP to aqueous-acetone soluble form(s) using the method of Kouri et al. (17). A 100 ul aliquot of the aqueous-acetone phase was added to 10 ml of scintillation fluid and counted in a liquid scintillation counter (Packard Instrument Co., Downers Grove, IL). The cpm were converted to nmol BP metabolites, using an external quench curve, an external ^{137}Cs standard, and the radiospecific activity of the tritium labeled BP. Control level activity resulting from incubation of BP for 24 hours in complete medium without cells was routinely subtracted from the levels with cells. For calculation of the enzymatic specific activity, total cellular protein was determined by the Lowry procedure, as modified by Oyama and Eagle (23). Results were expressed as nmol BP metabolized to an aqueous-acetone soluble form per mg total cellular protein.

DNA Synthesis Inhibition Assay

The inhibition of DNA synthesis in the cell lines to be examined was determined using a modification of the method described by Painter (18). Cells to be tested were plated at 25-100x 10^3 per 17 mm diameter multiwell dish (Falcon, Oxnard, CA) and allowed to attach for ~12 hr. Proximate carcinogens were dissolved in DMSO dried over molecular sieve type 3A (Matheson, Coleman & Bell Manufacturing Chemists, Norwood, OH) and the carcinogen-DMSO solution was added

to the complete medium to obtain the indicated final concentrations. The final DMSO concentration applied to each culture (including controls) was kept constant. After a 48 hr treatment period, the carcinogen containing medium was replaced with complete medium containing tritium labeled thymidine at a final concentration of 5 uCi/ml. After 2 hr of incubation, the thymidine containing medium was removed. The cells were rinsed with phosphate buffered saline, removed in 0.05% trypsin-o.o2% EDTA and collected on GF/C filters (Whatman LTD, England). The filters were rinsed two times with phosphate buffered saline and two times with 10% TCA. Then the filters were placed in scintillation vials and the DNA hydrolyzed by the addition of 1 ml 0.5N HCl and heated for 1 hr at 90^{o}C before addition of scintillation fluid and counting in a Beckman Model A LS9000 liquid scintillation spectrometer. In these samples tritium was counted with ~23% efficiency.

When the ultimate reactive form of a carcinogen was used in the DNA synthesis inhibition assay, all procedures were identical except that an acetone solution of the carcinogen was added to the serum-free medium covering the monolayer of cells and the treatment time was 2 hrs.

Cell Mediated Cytotoxicity Assays

Since the lethally irradiated cells cannot form colonies, the target cells were not separated from the metabolizing cells in this assay. The number of target cells present in the cocultivation plates was indirectly determined by trypsinizing a confluent culture of target cells alone and counting them with a hemocytometer or electronic counter (Coulter Corp., Hialeah, FL). The cocultivated cells were similarly trpsinized, suspended in culture medium, diluted, plated into 12 or more 60-mm diameter dishes at appropriate densities to obtain 40 to 60 colonies/dish, and allowed to develop into macroscopic colonies.

The cytotoxicity was determined from the cloning efficiency of target cells cocultivated with metabolizing cells divided by that of control cells, as defined in each experiment in each experiment, and is expressed as a percentage.

Mutagenicity Assay

To assay the cocultivated target cells for the frequency of B(a)P-induced mutations to 6-thioguanine resistance, cells were trypsinized and counted as described above and plated into 250-ml flasks to allow for expression of mutations at cell densities, which allowed surviving target cells to replicate without reaching confluence. (Sufficient numbers of flasks were used for each determination to ensure a minimum of 10^6 surviving target cells at the beginning of the expression period.) The cells were trypsinized, pooled, and replated into flasks one or more times to ensure logarithmic growth. The total length of the expression period was adjusted for each experimental determination to allow the cells to undergo at least 4.5 population doublings before selection was begun, i.e., 7 to 10 days (21).

At the end of the expression period, the cells were trypsinized, pooled, and 1 to 5 x 10^6 cells were plated into a selective medium at a density of 500 cells/sq cm. To determine their cloning efficiency at the time of selection, these cells were further diluted and plated at cloning densities into medium identical to selective medium but lacking 6-thioguanine. A reconstruction experiment with hypoxanthine (guanine) phosphoribosyltransferase Lesch-Nyhan cells (23) accompanying each determination indicated that the efficiency of recovery of 6-thioguanine-resistant colonies in the experiments reported here was $>85\%$. Selection was continued for 14 to 18 days with one refeeding. The frequency of mutations to 6-thioguanine resistance was calculated from the

probability of a mutant per dish using the Poisson Distribution Function, $P(0)-e^{-x}$ (17).

HPLC Analysis of B(a)P-DNA Adducts

Lethally irradiated metabolizing cells (Hs835T) cultured alone, confluent cultures of XP cells cultured alone, or lethally irradiated metabolizing cells cocultivated with confluent cultures of target XP cells were exposed for 48 hr to the indicated concentrations of tritiated B(a)P. At the end of the incubation period, the cells were removed from the flasks with trypsin: EDTA and lysed in 0.4% N-lauroylsarcosine, and the DNA was isolated as described previously (19). The DNA was further purified by CsCl density centrifugation (14) and the DNA fractions from these gradients were pooled and dialyzed. The number of B(a)P-DNA adducts in the extracted DNA was determined from the specific activity. The amount of DNA present was determined from the absorbance at 260 nm. The DNA was enzymatically degraded to nucleosides by the method of Yamasaki et al. (32), and the nucleoside adducts were chromatographed on Sephadex LH-20 (5). The methanol eluent containing the adducts was concentrated by evaporation under a gentle stream of nitrogen. The nucleosides with bound B(a)P residues were redissolved in 100 μl of methanol and separated with a 100-min concave gradient (NL2) of 30 to 60% methanol in water on a Spectra-Physics SP 8000 high-pressure liquid chromatograph fitted with a Water Associates $C_{18-\mu}$Bondapak column (3.9 x 300 mm) maintained at 50° (12). Fractions were collected at 1-min intervals, mixed with 10 ml scintillation fluid, and counted at approximately 45% efficiency on a Beckman Model A LS9000 liquid scintillation spectrometer equipped with automatic dpm calculation capability. The optical standards for cochromatographic comparison with the tritium-labeled cellular B(a)P-DNA adducts were synthesized by reacting 0.5 mg anti- or

syn-BPDE dissolved in 1 ml of acetone with 1 mg dGMP or salmon sperm DNA dissolved in 1 ml of water for 3 hr at 37^{o} at an acetone:water ratio of 1:1. The unreacted hydrocarbon was removed from the solution do dGMP by 2 extractions of ethyl acetate (1:1), the nucleotides were degraded to nucleosides with alkaline phosphatase (3 hr at 37^{o}), and the nucleoside adducts were further purified by LH-20 chromatography. After reaction of anti- or syn-BPDE with salmon sperm DNA, the unreacted hydrocarbon was removed with 2 extractions of ethyl acetate (1:1), the DNA was enzymatically hydrolyzed as for cellular DNA, and the nucleoside adducts were separated by LH-20 chromatography.

SECTION 4

RESULTS

Hydrocarbon-Metabolizing Activity Assay

Initial experiments were undertaken to determine the suitability of using the ability of cells to metabolize BP to an aqueous-acetone soluble product as a means of determining which cells might be suitable as feeder layers. However, before screening cells for their ability to metabolize BP to aqueous-acetone soluble products, we examined the hydrocarbon metabolizing assay with respect to time of exposure to BP and number of cells treated (see Figure 1). Since this assay was linear for at least 24 hr with cell numbers up to 8 x 10^5, we used a 24 hr treatment period and cell numbers no greater than 5 x 10^5.

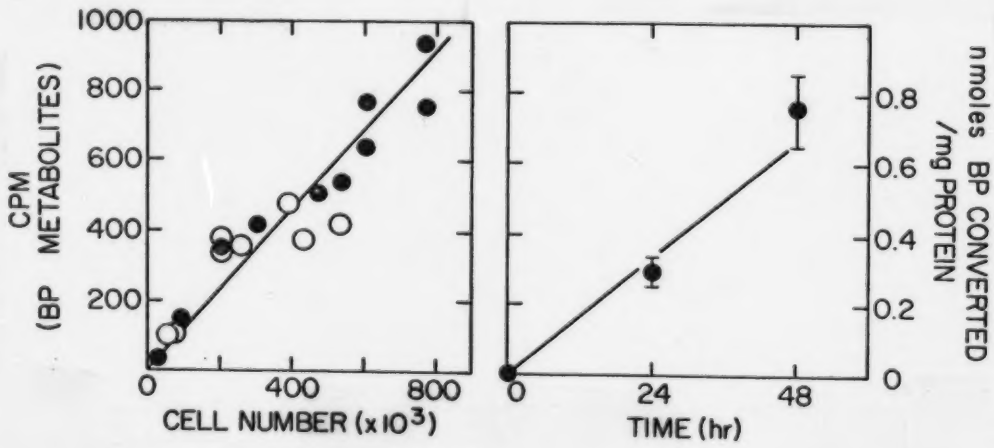

Figure 1. Hydrocarbon metabolizing activity as a function of cell number. In two experiments (●,○)PC-3 cells were plated at varying numbers in 60mm petri dishes and allowed to attach. All dishes were treated for 24 hr with 4 μM BP supplemented with ^{3}H-BP (specific activity, 0.5 mCi per μmole). At the end of the treatment period, the media was extracted with hexane, as described in Methods and Materials, and the radioactivity in the aqueous-acetone phase was determined.

Table 1 shows the results of screening 15 tumor-derived cell lines and 4 normal cell strains for hydrocarbon metabolizing activity. Hs835T cells served as a positive control and were run with every assay. Those cell lines above the dotted line in Table 1 showed consistently higher levels of metabolism and were considered good prospects for examination of cell-mediated cytotoxicity. Note

TABLE 1. HYDROCARBON-METABOLIZING ACTIVITY OF HUMAN CELLS

Cell Line Designation	nmoles BP Converted/ mg Protein/24 hr	Tissue of Origin and Diagnosis
Hs700T	27.8 ± 6.0[a]	Metastatic adenocarcinoma
Det 562	24.2 ± 13.5	Pharyngeal carcinoma
Hs703T	18.3 ± 3.2	Liver carcinoma
Hs835T	12.3 ± 3.6	From tumor produced by innoculation of Hs761T cells (kidney carcinoma) into nude mouse
A498	10.7 ± 0.4	Kidney carcinoma
A253	8.7 ± 4.4	Epidermoid carcinoma of the neck
A549	3.6 ± 0.4	Lung carcinoma
Hs696T	3.2 ± 0.4	Metastatic adenocarcinoma
Hs766	3.2 ± 0.4	Metastatic carcinoma of pancreas
Hs683T	2.4 ± 1.6	Glioma
HT1080	1.6 ± 0.8	Fibrosarcoma
JAR	0.8[b]	Choriocarcinoma
NF814.4[c]	0.8[b]	Normal skin fibroblasts
Hs695T	0.8[b]	Melanoma
A172	0.8 ± 0.4	Glioblastoma
FHS74INT	0.8 ± 0.4	Normal intestine
PC-3	0.8 ± 0.4	Bone marrow sample from patient with prostatic cancer
NF801.5[c]	0.4[b]	Normal skin fibroblasts
NF812.4[c]	0.4[b]	Normal skin fibroblasts

[a]Mean ± S.D.

[b]Only one determination

[c]Number to right of decimal indicates passage level.

the low activity of the normal cell strains. When confluent cultures of NF were exposed to BP at concentrations up to 30 μM for 48 hr and replated at low density for macroscopic colony formation, they showed little or no cytotoxic response (unpublished data). However, when confluent NF were cocultivated with increasing numbers of lethally irradiated Hs835T cells and treated for 48 hr with 4 μM BP, their survival (as reflected in colony-forming ability) was

reduced to <10% of control cells incubated with 4 μM BP in the absence of metabolizing cells (Figure 2). Since the lethally irradiated metabolizing cells in the absence of BP do not produce or release compounds which are cytotoxic to the target cells (unpublished data), this suggests that the metabolizing cells are producing cytotoxic metabolites from BP.

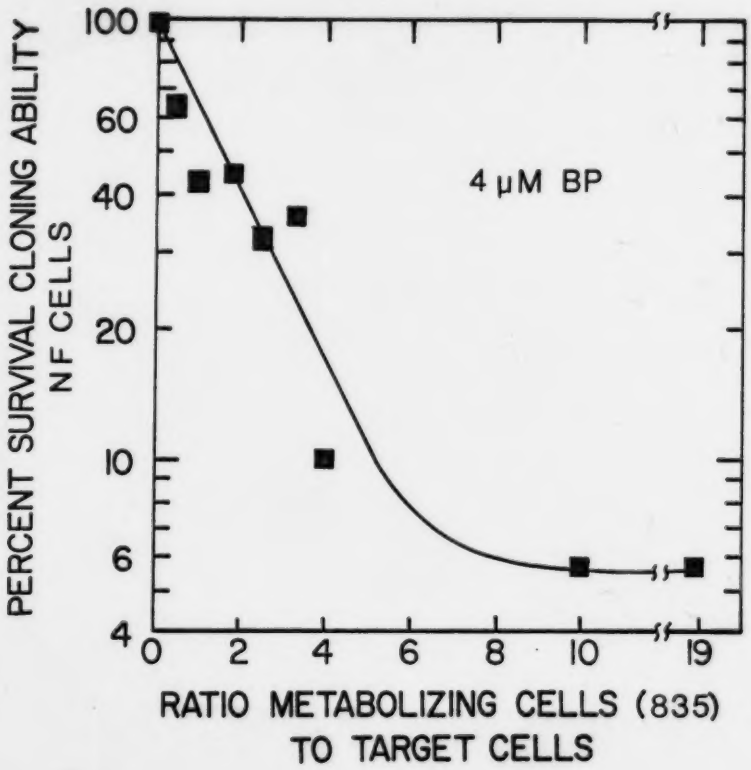

Figure 2. Cytotoxic effect of 4 μM BP as a function of the ratio of metabolizing cells (Hs835T) to target cells (NF). NF were grown to confluence in 34 mm dishes. The number of cells was determined, and lethally irradiated Hs835T cells were seeded on top of the target cells at the indicated ratios and also into corresponding sets of dishes without target cells. After a 48 hr exposure to 2 ml of medium containing 4 μM BP, the cultures were replated at cloning densities.

DNA Synthesis Inhibition Assay

To identify cells capable of activating chemical carcinogens which cannot be examined directly for metabolism because they are not available in radioactive labeled form or because the metabolites cannot be separated from the parent molecule by these procedures, we examined the ability of cells to activate them during a 48 hr exposure into forms capable of inhibiting DNA synthesis. As a preliminary step we determined the parameters of tritiated thymidine incorporation in exponentially-growing untreated cells. The results (Figure 3) indicated that at a density of $\lesssim 60 \times 10^4$ cells/dish incorporation was linear for at least 2 hr. Therefore, 2 hr incubation and 2.5 to 10×10^4 cells/dish were used in all subsequent assays to insure comparable results among different cell lines.

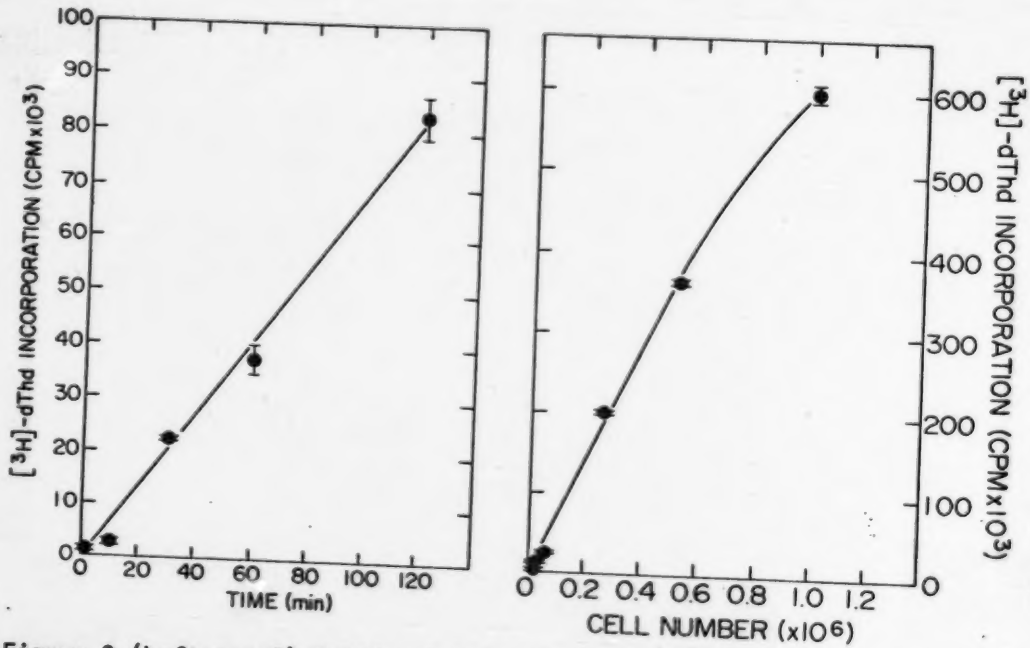

Figure 3.(Left panel) Tritium labeled thymidine incorporation into cellular DNA as a function of time. Exponentially growing untreated Hs703T cells (0.1×10^6) were exposed to tritiated thymidine at a concentration of 5 μCi/ml for the indicated times. The level of incorporation into cellular DNA was determined as described in Materials and Methods. (Right panel) Incorporation as a function of cell number.

To determine whether this assay was sensitive enough to show a response at low concentrations of carcinogen, but concentrations at which cells show a cytotoxic or mutagenic response, we examined the DNA synthesis inhibition in NF after a 2 hr treatment with BPDE 1, the ultimate reactive form of BP. The results (Figure 4) show that at a concentration that would result in 37% survival, BPDE 1 decreased DNA synthesis to 40% that of the control. We showed previously that the level of DNA binding for this level of cytotoxicity is ~8 BPDE 1 residues per 10^6 nucleotides (23). The Hs703T cells which were tested at the same time showed a similar response. As a positive control, we examined two

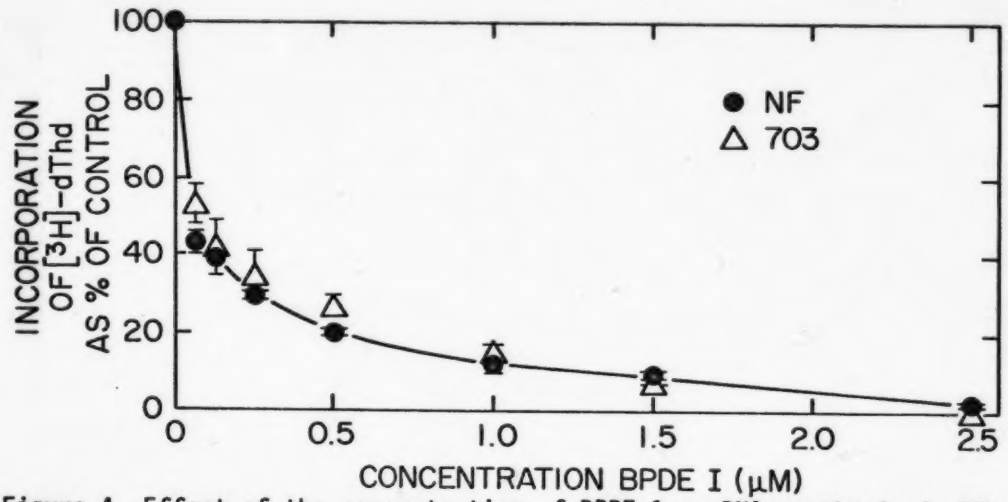

Figure 4. Effect of the concentration of BPDE 1 on DNA synthesis in NF and Hs703T cells. NF or Hs703T cells were treated for 2 hr with various concentrations of BPDE 1. At the end of the treatment period the inhibition of DNA synthesis was determined as described in Materials & Methods. The results for NF (●) and Hs703T cells (Δ) were plotted as a function of the treatment concentration of BPDE 1.

cell lines that we had already shown were capable of metabolizing BP (Hs835T and Hs703T) and one cell strain that cannot (XP12BE, unpublished data), for the effect of BP on cellular DNA synthesis. The results (Figure 5) showed that inhibition of synthesis was correlated with the capability of the cells to metabolize BP to a biologically reactive form (10). We concluded that the DNA

synthesis inhibition assay could not only reflect metabolism to reactive forms, but also was sensitive at biological levels.

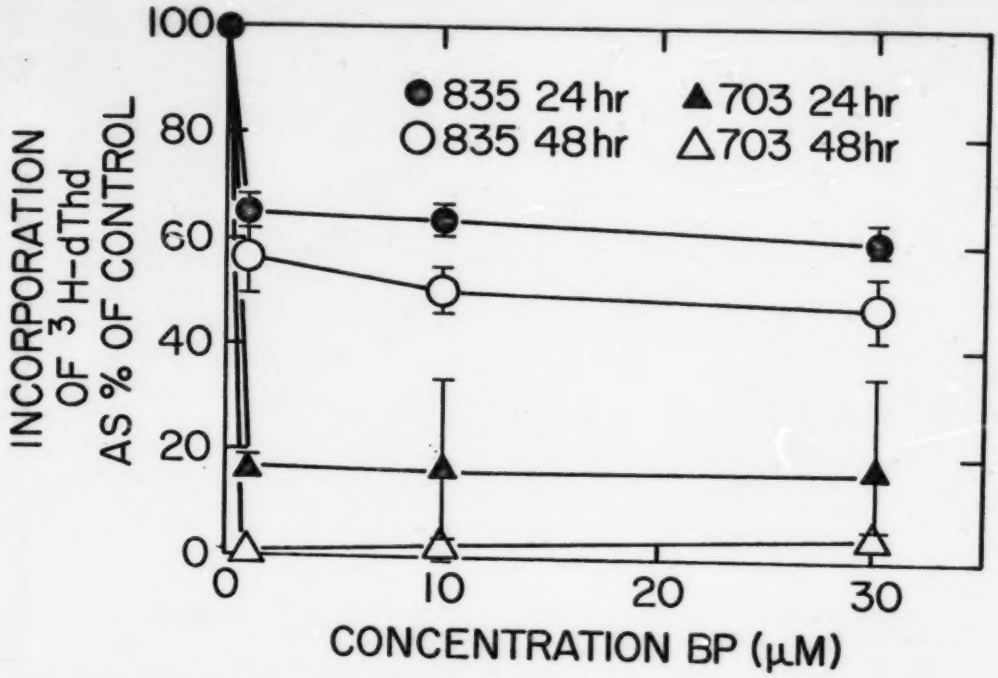

Figure 5. The effect of the concentration of BP and time of exposure on cellular DNA synthesis in Hs835T cells and Hs703T cells. Hs835T cells and Hs703T cells were treated for 24 or 48 hr with increasing concentrations of BP. At the end of the treatment period inhibition of DNA synthesis was determined as described in Materials and Methods. The results for Hs835T cells (●, 24 hr; ○, 48 hr) and Hs703T cells (▲, 24 hr; △48 hr) were graphed as a function of BP concentration.

We examined the capability of human cells to metabolize other compounds using this assay. Four classes of compounds were studied: aromatic amines, nitrosamines, heterocyclic hydrocarbons, and other polycyclic hydrocarbons. For the polycyclic hydrocarbon study, we used Hs703T cells, a line that we showed had a high capacity for metabolizing polycyclic hydrocarbons, and three structurally related compounds, B(b)F, B(j)F, and B(k)F. The results of the DNA synthesis inhibition assay (Figure 6A) suggested that Hs703T cells metabolized the compounds to biologically reactive forms. To confirm this, we examined them

further using the cell-mediated cytotoxicity assay with XP as target cells (Figure 6B) and found a concentration-dependent decrease in the survival of the target XP cells with B(b)F up to 0.75 μM. These results confirmed that cytotoxic metabolites were being produced by the metabolizing cells.

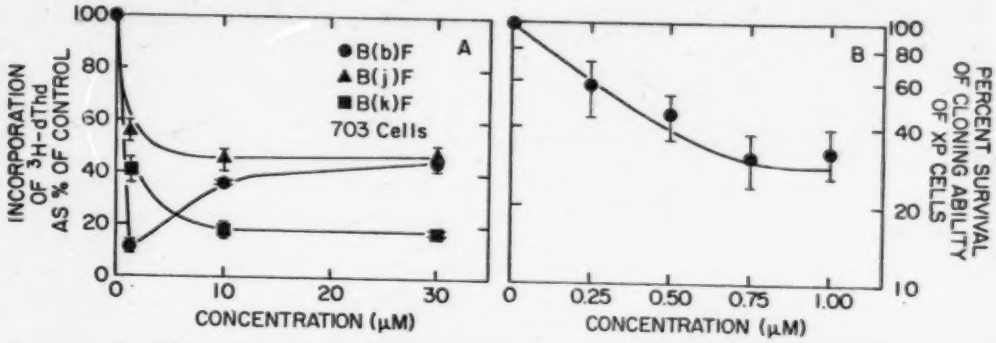

Figure 6. (A) Comparison of the effect of concentration of B(b)F, B(j)F, and B(k)F on cellular DNA synthesis in Hs703T cells. The inhibition of DNA synthesis was determined after 48 hr treatment with various concentration of B(b)F (●), B(j)F (▲), or B(k)F(■). (B) The effect of the concentration of B(b)F on the survival of the cloning ability of target XP cells cocultivated with Hs703T cells. Lethally irradiated Hs703T cells were added to confluent cultures of XP cells. After a 48 hr exposure to the indicated initial concentrations of B(b)F, the cytotoxicity was determined as described in Figure 2.

To identify cells capable of metabolizing aromatic amines, we examined the inhibition of DNA synthesis by AABP, AAF, and MBOCA in candidate metabolizing cells. The results (Figure 7A) suggested that Hs700 cells are capable of activating this class of compounds. This conclusion was confirmed by the results of the cell-mediated cytotoxicity assay (Figure 7B) which showed a decreased survival of the XP target cells with increasing concentrations of MBOCA up to 5μM.

The cell line Hs703T was also tested for its ability to activate the heterocyclic compounds azapyrene, azachrysene, and azabenzanthracene. The results of DNA synthesis inhibition experiments (Figure 8A) suggested that it can activate these compounds. Cell lines were also tested for their ability to activate the

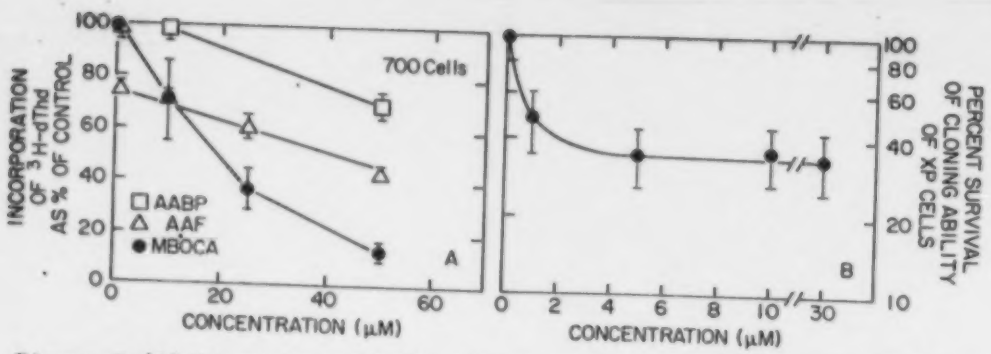

Figure 7.(A) Comparison of the effect of the concentrations of three aromatic amines: AABP (□), AAF (△), and MBOCA (●), on cellular DNA synthesis in Hs700T cells. Inhibition of DNA synthesis in Hs700T cells was determined as described after 48 hr treatment with various concentrations of AABP,AAF, and MBOCA. (B)The effect of the concentration of MBOCA on the survival of the cloning ability of target XP cells cocultivated with Hs700T cells. Lethally irradiated Hs700T cells were added to confluent cultures of XP cells. After a 48 hr exposure to the indicated initial concentrations of MBOCA, the cytotoxicity was determined as for Figure 2.

nitrosamines, DEN and DMN. Results with the two of the cell lines tested suggested that both compounds could be activated. DEN producted greater DNA synthesis inhibition than DMN at similar concentrations (Figure 8B).

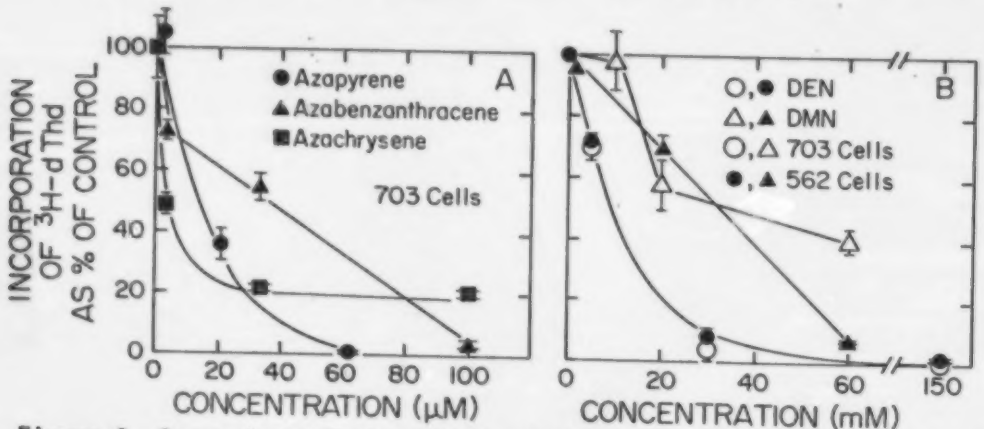

Figure 8. Comparison of the effect of the concentration of azapyrene(●) azachrysene (■), and azabenzanthracene (▲) on cellular DNA synthesis in Hs703T cells. (A), or DEN and DMN in Det562 and Hs703T cells, (B) afrer a 48 hr treatment.

Effect of Length of Exposure. The data in Figure 9 also illustrate that if the exposure of the metabolizing and target XP cells to B(a)P was allowed to continue for 48 hr the cytotoxic effect of B(a)P on the XP cells was greatly increased over that of 24 hr. However, both exposure periods display the plateau in cytotoxic response at higher concentrations of B(a)P. This further supports the suggestion that one or more of the B(a)P-metabolizing enzymes producing the cytotoxic metabolite(s) is becoming saturated or inhibited. The control for each time period was target XP cells cocultivated with metabolizing cells at

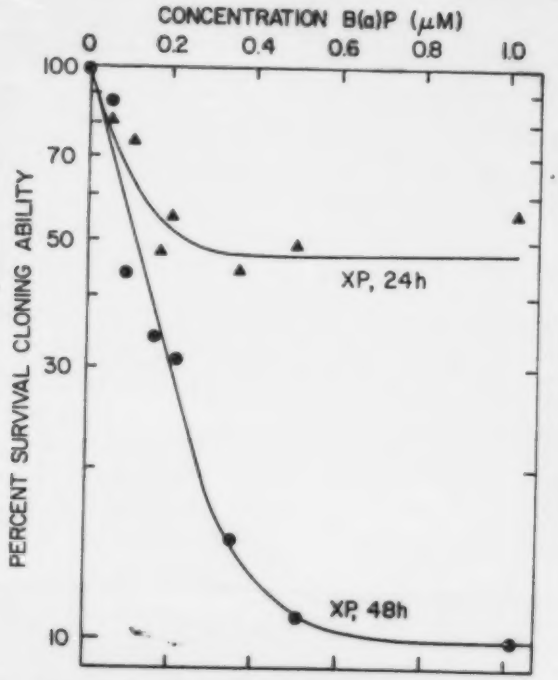

Figure 9. The effect of the concentration of B(a)P and time of exposure on the percentage of survival of the cloning ability of cocultivated target XP12BE cells. Lethally irradiated Hs835T cells were added to 35-mm plastic dishes containing confluent cultures of XP12BE cells such that the ratio of attached metabolizing cells:target cells was 1:1. After a 24-(▲) or 48-hr(●)exposure to 2 ml of culture medium containing the indicated initial concentrations of B(a)P, the cells were replated at cloning density as described in Materials and Methods.

the same ratio as in the experimental dishes but without B(a)P treatment. Because of the significantly greater response at 48 hr this exposure time was routinely

Experiment	Expression time (days)	B(a)P concentration (μM)	Dishes without mutants/total no. of dishes	Cloning efficiency at selection (%)	Mutant frequency / 10^6 clonable cells
1[a]	7	0	147/150 (1.6)[b]	16	15
	7	0.16	152/155 (1.6)	18	11
	7	0.23	119/124 (1.3)	15	30
	9	0.31	110/128 (1.3)	15	100
2[c]	8	0	127/127 (1.3)	11	<11
	8	0.25	176/180 (1.9)	6	34
	8	0.34	139/143 (1.5)	8	43
3[d]	8	0	153/171 (4.7)	12	31
	8	0.1	154/176 (4.8)	7.5	60
	8	0.2	117/171 (4.7)	7.5	171
	10	0	150/180 (5.0)	13	51
	10	0.1	63/87 (2.4)	10.5	116
	10	0.2	44/65 (1.8)	10	150

[a] Ratio of metabolizing to target cells, 1.3:1.
[b] Numbers in parentheses, total number of selected cells (in millions).
[c] Ratio of metabolizing to target cells, 2.5:1.
[d] Ratio of metabolizing to target cells, 0.75:1.

Table 2. B(a)P Induction of 6-Thioguanine-Resistant Colonies in Target XP12BE Cells Cocultivated With Metabolizing Cells.

Lethally irradiated Hs835T cells were added to 250-ml flasks containing confluent cultures of XP12BE cells. After a 48-hr exposure to 19 ml of culture medium containing the designated concentrations of B(a)P, the cells were replated at low cell density and allowed to undergo expression for 7 to 10 days. At the end of the expression period, the cells were trypsinized, pooled, and plated into 6-thioguanine-containing medium. (For Experiments 1 and 2, 1.3 to 2 x 10^6 cells were plated at 10,500 cells/60-mm-diameter dish; for Experiment 3, 1.8 to 5 x 10^6 cells were plated at 27,500 cells/100-mm dish.) The frequency of 6-thioguanine-resistant colonies was calculated from the probability of a mutant per dish calculated from $P(0)=e^{-x}$, Poisson Distribution Function ($_{30}$). This frequency was corrected to mutation frequency per 10^6 clonable cells using the cloning efficiency of the cells at the time of selection.

HPLC Analysis of the B(a)P-DNA Adducts

The B(a)P-induced increase in the frequency of 6-thioguanine-resistant colonies of target cells suggests interaction of B(a)P metabolites with cellular DNA. To investigate the extent and nature of this interaction, we determined the number of B(a)P-DNA adducts that were formed during a 48-hr exposure period of 60 x 10^6 cocultivated metabolizing cells and target cells at a ratio of 3:1 to tritiated B(a)P at 3 concentrations, namely, 0.1, 0.2, and 0.7 μM. The number of B(a)P adducts per 10^7 DNA nucleotides were found to be, respectively, 4.5, 8.8, and 20. These results confirmed that metabolites of B(a)P were binding to the cellular DNA and showed a direct relationship between the concentration of B(a)P in the medium and the number of B(a)P-DNA adducts formed.

used for all subsequent cell-mediated cytotoxicity or mutagenicity studies. Longer exposure periods were not used because in 48 hr greater than 90% of the hexane-extractable B(a)P was found to have been removed from the medium at concentrations up to 2 μM B(a)P. Since most of the B(a)P present in the medium does get metabolized by the cells under the conditions used, the concentration of B(a)P as well as the volume of B(a)P-containing medium were found to be critical.

Mutagenicity as Evidence of Activation of B(a)P by Human Cells

Using the optimal cocultivation conditions (48 hr exposure to B(a)P at metabolizing cell:target cell ratios of 0.6:1 or greater), we assayed the XP cells for B(a)P-induced mutations. The results of 3 separate experiments are shown in Table 2. In each experiment, the frequency of 6-thioguanine-resistant colonies observed in the B(a)P-treated populations increased with increasing concentrations of B(a)P. The induced mutation frequencies observed are consistent with those we observed in XP cells treated with concentrations of anti-BPDE which resulted in similar toxicities (23). We have not assayed representatives of these 6-thioguanine-resistant colonies for hypoxanthine (guanine) phosphoribosyltransferase activity, but a series of 6-thioguanine-resistant colonies isolated from previous experiments using identical selection conditons, with UV radiation or N-acetoxy-2-acetylaminofluorene as the mutagen, showed enzyme activities to be less than 8% of control.

DNA extracted from 60 x 10^6 XP cells alone, treated for 48 hr with 0.7 μM tritiated B(a)P, showed radioactivity of less than 10% of that associated with DNA from the cocultivated cultures. HPLC analysis showed no radioactivity eluted in the B(a)P-DNA adduct region. The low level of radioactivity associated with this DNA made further analysis difficult. However, since the exposure to this concentration of B(a)P did not produce a cytotoxic effect in the XP cells cultured alone, we did not pursue any further the nature of this low level of radioactivity.

DNA extracted from 60 x 10^6 lethally irradiated metabolizing cells treated for 48 hr with 0.7 μM tritiated B(a)P, showed a binding level identical to that found in the DNA of identically treated cocultivated cultures at a metabolizing cell:target cell ratio of 3:1. This suggests strongly that the level of binding to the DNA of the cocultivated XP cells is similar, if not identical, to that of the metabolizing cells. To further examine this in a subsequent experiment, lethally irradiated metabolizing cells cultured alone and cocultivated metabolizing and target XP cells, at a reduced ratio of 1:1, were treated for 48 hr with 0.7 μM tritiated B(a)P, and the DNA was extracted. Since the metabolizing cell:XP cell ratio was substantially reduced (from 3:1 to 1:1), the DNA from the XP cells represented a higher percentage of the total DNA isolated from the cocultivated cells and would, therefore, change the apparent overall binding level if the binding to the DNA of the XP cells were significantly different from that of the metabolizing cells. In fact, the number of B(a)P-DNA adducts formed in the metabolizing cells alone was the same as that in the cocultivated cells, i.e., $25/10^7$ DNA nucleotides. These data are consistent with the interpretation that the level of binding to the DNA of the cocultivated XP cells is very similar, if not identical, to that of the metabolizing cells. However, we are presently developing techniques to separate the target and metabolizing

cells in sufficient quantity so that, after treatment in cocultivated cultures, we can determine the level of binding to each cell type.

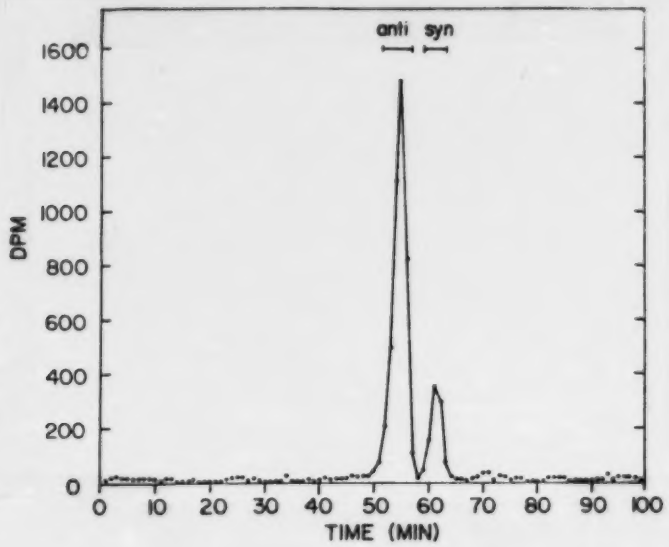

Figure 10. HPLC elution profiles of B(a)P-DNA adducts formed in cocultivated cultures of lethally irradiated Hs835T cells and XP12BE cells incubated with tritium-labeled B(a)P. Lethally irradiated Hs835T cells were added to 250-ml flasks containing confluent cultures of XP12BE cells at a metabolizing cell:target cell ratio of 3:1. After a 48-hr exposure to 19 ml of culture medium containing 0.7 μM tritium-labeled B(a)P, the cells were removed from the flasks and pooled, and the DNA was isolated and analyzed as described in Materials and Methods. Bars, the elution positions of deoxyguanosine adduct standards synthesized with anit- and syn-BPDE.

To identify these B(a)P-DNA adducts, we used HPLC to analyze those resulting from treatment with 0.7 μM tritiated B(a)P. Figure 10 shows the HPLC elution profile obtained. This is the same profile obtained when the DNA adducts from the metabolizing cells alone were analyzed. The major peak, 82% of the radioactivity associated with the adducts, cochromatographed with the optical standard adduct formed between deoxyguanosine and anti-BPDE. This cell-mediated B(a)P-DNA adduct also cochromatographed with the adduct formed in normal diploid human skin fibroblasts exposed for 2 hr to anti BPDE (23). The minor radioactive peak in

Figure 10 cochromatographed with the optical standard formed between deoxyguanosine and syn-BPDE. These results are consistent with reports on B(a)P-DNA adducts formed in human explant tissue from lung (24), colon (25), esophagus (26), and bronchus (27), as well as cells derived from human lung carcinoma A549 (28), in which the N^2-deoxyguanosine-anti-BPDE adduct was the major adduct observed.

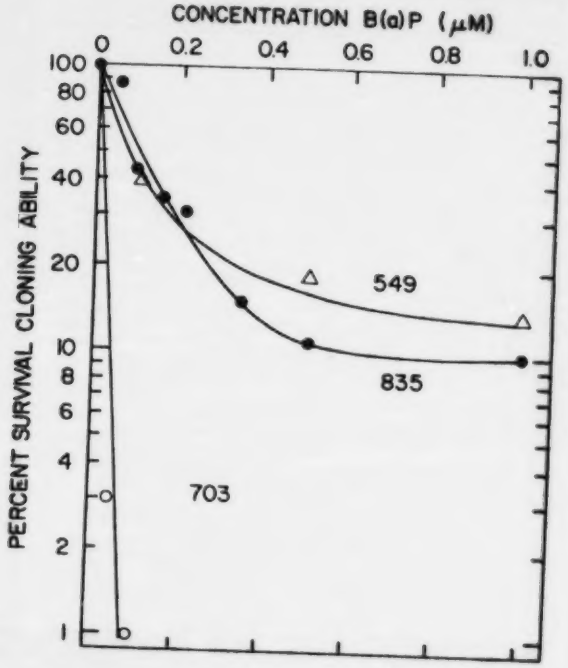

Figure 11. Comparison of the ability of 3 human epithelial cell lines, derived from kidney carcinoma (●), liver carcinoma (○), and lung carcinoma (△), to produce a cytotoxic effect in target XP12BE cells during a 48-hr exposure to various concentrations of B(a)P. The attached metabolizing cell:target cell ratio was 1:1. Procedures used are as described in the legend for Figure 9 from which the Hs835T data were taken.

Activation of B(a)P by Other Human Epithelial Tumor Cell Lines

To determine whether the system described here could be successfully used with other human cell lines as metabolizing cells, 2 human epithelial tumor cell lines which also exhibited relatively high levels of B(a)P metabolism

were tested in the cell-mediated cytotoxicity assay. Figure 11 compares the cytotoxicity induced by B(a)P in target XP cells cocultivated with cells derived from liver carcinoma or from lung carcinoma with that found with the kidney carcinoma-derived cells used in the above studies. These preliminary results suggest that the liver carcinoma cells produce more cytotoxic B(a)P metabolites than do either of the other 2 cell lines. More importantly, they demonstrate that this cell-mediated system works successfully with other metabolizing cell lines.

Recent studies show that benzo(b)fluoranthene and benzo(k)fluoranthene are converted by Hs703 cells to mutagenic metabolites for XP target cells. In addition, we found that these metabolites have the ability to neoplastically transform human fibroblasts using our recently published assay (31). More complete data on transformation will be available for the final report on this grant.

SECTION 5

DISCUSSION

In this report, established human epithelial cell lines and fibroblast strains were monitored by two procedures for their ability to metabolize various carcinogens. Each of the techniques served as an excellent prescreening method for identifying cells capable of carcinogen activation. In the first procedure, which identified polycyclic hydrocarbon metabolizing cells, the conversion of tritiated BP to aqueous-acetone soluble products was determined using the method of Kouri et al. (17). Although this assay does not specifically determine the enzymatic activity responsible for production of BPDE 1, the metabolite most likely to be the carcinogenic form of BP, the assay was useful because it reflected, in a qualitative way, the ability of the cells to produce cytotoxic metabolites. Cells with very low metabolizing activity, such as NF, showed little or no cytotoxic response when exposed to BP at concentrations up to 30 uM for 48 hr. However, when the NF, cocultivated with lethally irradiated Hs835T cells, which had 20 times the hydrocarbon-metabolizing capability, were exposed to 4 uM BP for 48 hr, their survival was reduced to <10% that of the control. A similar observation was made with XP cells which showed no cytotoxic response to BP unless cocultivated with a metabolizing cell line, such as Hs835T, Hs703T, or A549 (10). In fact, there was qualitative correlation between the cytotoxic response of XP cells and the level of enzymatic activity of the metabolizing cells in that Hs703T > Hs835T > A549. In other studies with the Hs835T cocultivated with the XP target cells we showed that there was a BP concentration-dependent increase in the frequency of 6-thioguanine resistant colonies and in the level of metabolite binding to cellular DNA (Table II). The predominant DNA adduct resulted from the reaction between BPDE 1 and N^2-deoxyguanosine (Figure 10).

This is consistent with the adducts observed in human tissue explants treated with BP (24-27). Thus, the biological results (cytotoxicity, mutagenicity and DNA binding) we observed using the cell-mediated system suggest that the hydrocarbon-metabolizing activity assay is a very useful prescreen for identifying human cells that metabolize BP to biologically active form(s).

The second method, the inhibition of cellular DNA synthesis after treatment with carcinogens, was selected as a prescreen because we wanted an assay that reflected interaction of carcinogens with DNA. As discussed by Painter (28), DNA synthesis inhibition does not always have to reflect an irreversible interaction with DNA. However, we found that all the cells that showed carcinogen-dependent inhibition of DNA synthesis, which were subsequently tested for production of mutagenic and/or cytotoxic metabolites using the human cell-mediated assay, proved to be able to produce such metabolites. These results suggest that this assay is also a very useful prescreen for identifying cells with the potential for metabolizing carcinogens. In addition, the assay appears to be very sensitive. For example, as few as 8 residues of BPDE 1 per 10^6 DNA nucleotides reduced DNA synthesis to 40% of normal. Although as discussed by Painter (28), the apparent sensitivity of the DNA synthesis assay may vary depending upon the cells being tested, the relative cytotoxic response obtained with BP in target XP cells cocultivated with the metabolizing cells Hs835T or Hs703T (10) correlated with their relative BP metabolizing capability as predicted from the DNA synthesis inhibition assay.

In conclusion, the assay systems described here use concentration-dependent cytotoxicity and mutagenicity in human cells as sensitive biological responses to human epithelial cell activation of B(a)P. The use of human carcinoma cell lines which are characterized by constant high levels of B(a)P-metabolizing activity allowed the optimization of assay conditions, which was not possible with the primary epithelial cell-mediated system reported by Curren et al. (29).

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